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Elevated tRNA$_i^{Met}$ Synthesis Can Drive Cell Proliferation and Oncogenic Transformation

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SUMMARY

Characteristics of transformed and tumor cells include increased levels of protein synthesis and elevated expression of RNA polymerase (pol) III products, such as tRNAs and 5S rRNA. However, whether deregulated pol III transcription contributes to transformation has been unclear. Generating cell lines expressing an inducible pol III-specific transcription factor, Brf1, allowed us to raise tRNA and 5S rRNA levels specifically. Brf1 induction caused an increase in cell proliferation and oncogenic transformation, whereas depletion of Brf1 inhibited transformation. Among the gene products induced by Brf1 is the tRNA$_i^{Met}$ that initiates polypeptide synthesis. Overexpression of tRNA$_i^{Met}$ is sufficient to stimulate cell proliferation and allow immortalized fibroblasts to form foci in culture and tumors in mice. The data indicate that elevated tRNA synthesis can promote cellular transformation.

INTRODUCTION

Cell-cycle progression requires attainment of adequate mass (Brooks, 1977; Johnston et al., 1977). Since most of a cell's dry mass is protein, the rate of protein accumulation is a fundamental determinant of cell growth and proliferation rates (Zetterberg and Killander, 1965). This may explain why elevated protein synthesis is strongly linked to cancer (Bjornsti and Houghton, 2004; Mamane et al., 2004; Pandolfi, 2004; Ruggero and Pandolfi, 2003). Components of the translation machinery are commonly deregulated in cancers, and in several cases the oncogenicity of these events has been established through genetics or the use of animal models (Bjornsti and Houghton, 2004; Mamane et al., 2004; Pandolfi, 2004; Ruggero and Pandolfi, 2003). Tumours display both quantitative and qualitative changes in protein expression, with preferential translation of some mRNAs encoding growth-promoting proteins (Mamane et al., 2004; Pandolfi, 2004).

Translation depends on tRNA and 5S rRNA, short abundant transcripts that are made by pol III. Overexpression of these and other pol III products has been observed in many types of transformed cells such as ovarian carcinomas (Winter et al., 2000; White, 2004). A partial explanation for this abnormal elevation is that the pol III-specific transcription factor TFIIIB is bound and activated in healthy cells by the tumor suppressors RB and p53 (Felton-Edkins et al., 2003b; White, 2005). Since the function of p53 and/or RB is compromised in most cancers (Evan and Vousden, 2001; Sherr and Weinberg, 2000), TFIIIB may be released from restraint in a high proportion of malignancies. Evidence for this has come from several studies. For example, the ability of RB to inhibit pol III output is compromised by cancer-associated mutations (Felton-Edkins et al., 2003b; White, 2004; White et al., 1996). Pol III transcription is stimulated by viral oncoproteins that bind and neutralize RB (Larminie et al., 1999; Sutcliffe et al., 1999; White et al., 1996; Felton-Edkins and White, 2002). Furthermore, inactivation of RB through deregulated phosphorylation releases TFIIIB and raises pol III output (Scott et al., 2001). Similarly, genetic mutation of p53 or overexpression of the oncoproteins E6 and Mdm2 also result in elevated pol III transcription (Morton et al., 2007; Stein et al., 2002). In addition, TFIIIB is bound and activated by the proto-oncogene product c-Myc, which is overexpressed in many cancers (Felton-Edkins et al., 2003b; Gomez-Roman et al., 2003). TFIIIB activity is also stimulated through direct phosphorylation by Erk kinases, which are abnormally active in ~30% of human tumors due to oncogenic mutations in Ras or Raf that lie upstream of Erk in the MAP kinase cascade (Felton-Edkins et al., 2003a). Clearly, TFIIIB is subject to powerful regulatory influences that frequently go awry during cell transformation.

RB, p53, Erk, and c-Myc all have multiple targets and pleiotropic effects. It could therefore be argued that elevated pol III transcription is a side effect of their deregulation, perhaps with little impact or significance. However, such an argument cannot explain an unrelated mechanism that also raises pol III output in some transformed cell types. TFIIIC, a factor essential for tRNA and 5S rRNA synthesis, is induced at both the mRNA and protein levels in cells transformed by SV40, polyomavirus, and Epstein Barr virus (Felton-Edkins et al., 2006; Felton-Edkins and White, 2002). This is not a secondary response to accelerated proliferation because TFIIIC levels are not influenced by growth factor availability or cell-cycle arrest (Scott et al., 2001; Winter et al., 2000). Similar deregulation was found in biopsies from ovarian cancer patients (Winter et al., 2000). The DNA-binding activity

of TFIIIC was measured in extracts of tissue from nine individuals with grade 2 or 3 ovarian carcinomas; in every case, the tumor had higher TFIIIC activity than healthy ovarian tissue from the same patient (Winter et al., 2000). RT-PCR revealed that the tumors overexpress mRNAs encoding all five subunits of TFIIIC, while control mRNAs remained at normal levels (Winter et al., 2000). The fact that all five subunits of TFIIIC were elevated in each case examined makes it extremely unlikely that this is a random effect. These observations provided evidence that a pol III-specific factor is overproduced in a human cancer. Since TFIIIC is dedicated exclusively to pol III transcription, this implies a specific drive to raise pol III output as the cancer develops.

Despite significant progress in understanding the molecular basis of pol III activation in cancers, the phenotypic consequences have remained a matter of conjecture. Here we address this issue directly by specifically inducing pol III transcripts independently of the many confusing genetic and epigenetic changes that normally accompany cell transformation. To this end, we have examined the effects of raising levels of the pol III-specific transcription factor Brf1, an essential subunit of TFIIIB. Induction of Brf1 results in a highly specific elevation of tRNA and 5S rRNA expression in both fibroblast and ovarian epithelial cell lines. This is accompanied by a substantial increase in the rate of protein synthesis. Under these conditions, cell-cycle progression and cell proliferation are stimul markedly. Furthermore, cells treated in this way manifest ch teristic features of oncogenic transformation, including fo formation, loss of anchorage dependence, and tumorigenici when injected into mice. The response to Brf1 urtailed by using RNAi to achieve partial knockdown pol ubunit. Furthermore, overexpression of the pol III scribe RNA^{Met} gene can itself be sufficient to drive roli and tumors in mice. The data suggest at pol I tivation can have profound and unexpected c uences tha contribute significantly toward cance

RESULTS

Induction of Brf1 mula Pol III Transcription Spec

TFIIIB is nec and cient ecruit pol III to its genetic templates lass tis et 0). It is composed of three essenti subunit TBP, Bdp , and either Brf1 or the related subuni 2 (S Hernandez, 2002). To stimulate pol III transcr specifically, we created stably transfected lines of immortali mouse embryonic fibroblasts carrying cDNA encoding Brf1 (MEFs). A Tet-Off system was used in which expression of Brf1 mRNA can be induced by withdrawing doxycycline from the culture medium (Figure 1A). This results in a modest increase in the level of Brf1 protein (Figure 1B). TBP and Bdp1 levels show little or no change under these circumstances. Nevertheless, Brf1 induction raises expression of 5S rRNA and various tRNAs, an effect not seen when control clones (vec.MEFs) carrying empty vector are treated in the same way (Figure 1C and Figure S1 available online). Brf1 also stimulates expression of pol III transcripts from B2 short interspersed repeats (SINEs), which are tRNA-derived pseudogenes (Daniels and Deininger, 1985). However, the response is highly selective and many other pol III products are not induced. For example, levels of U6, 7SK, and MRP RNAs are not elevated, which is consistent with the fact that expression of these products requires Brf2 instead of Brf1 (Schramm et al., 2000). Thus, chromatin immunoprecipitation (ChIP) shows little or no binding of Brf1 at 7SK genes, even when it is overexpressed (Figure 1D). In contrast, Brf1 is clearly detected at tRNA and 5S rRNA genes and its occupancy increases after removal line. ChIP also confirms that Brf1 induction stim s recru t of pol III to tRNA and 5S, but not 7SK gene contrast to K, both Brf1 and pol III are recruited to 7S gene lowing d cycline withdrawal (Figure 1D). Degra on of surp tran pt might account for the fact that 7S NA do not inc nder these circumstances (Figure 1C

The data show that rf1 i n can e a highly specific increase in expre n of a su of products. Further evidence of spe is provided pol I-dependent rRNAs (5.8S, 18S, a 8S) the pol II-dependent mRNAs encoding TBP, Bdp1, TK, and P0, all of which remain at near consta s (Figures 1A 1C). Expression of unprocessed prec or rRNAs is also unaltered (Figure S2).

Ind ion of Brf an Stimulate Protein Synthesis

Since As an S rRNA are essential components of the protein s machinery, their elevated expression following induction might influence the rate of translation. That this is th as shown by measuring incorporation of radioactive ino acids into newly synthesized polypeptides (Figure 2A). Protein synthesis had risen substantially after 3 days of Brf1 nduction, an effect not seen when empty vector control clones were treated in the same way. Accordingly, the overall accumulation of protein is significantly higher with elevated Brf1 (Figure 2B). We conclude that the specific induction of Brf1, and hence tRNA and 5S rRNA, is accompanied by a marked increase in protein synthesis. This can be explained if Brf1 is controlling production of something that is rate limiting for translation.

Elevated translational activity is not expected to increase synthesis of all proteins uniformly; preferential induction is often seen from mRNAs with complex secondary structures in their 5′-untranslated regions (Koromilas et al., 1992; Mamane et al., 2004). Examples are provided by cyclin D1 and c-Myc, both of which are subject to translational regulation (Mamane et al., 2004). Induction of Brf1 produced a selective increase in expression of these proteins without altering the levels of their mRNAs (Figure 2C). We conclude that elevated Brf1 can trigger both qualitative and quantitative changes in protein synthesis.

Induction of Brf1 Can Promote Cell-Cycle Progression and Proliferation

Despite the clear increase in protein production, cell volume showed little change in response to Brf1. Indeed, normalization to cell number revealed that the protein content of each cell had risen only marginally (Figure 2D). This clearly suggested a proliferative response, such that the accumulation of protein is balanced within a population by an increase in cell number. Precedent for such effects is provided by certain translation factors, activation of which can be sufficient to trigger cell proliferation, presumably due to elevated rates of protein synthesis

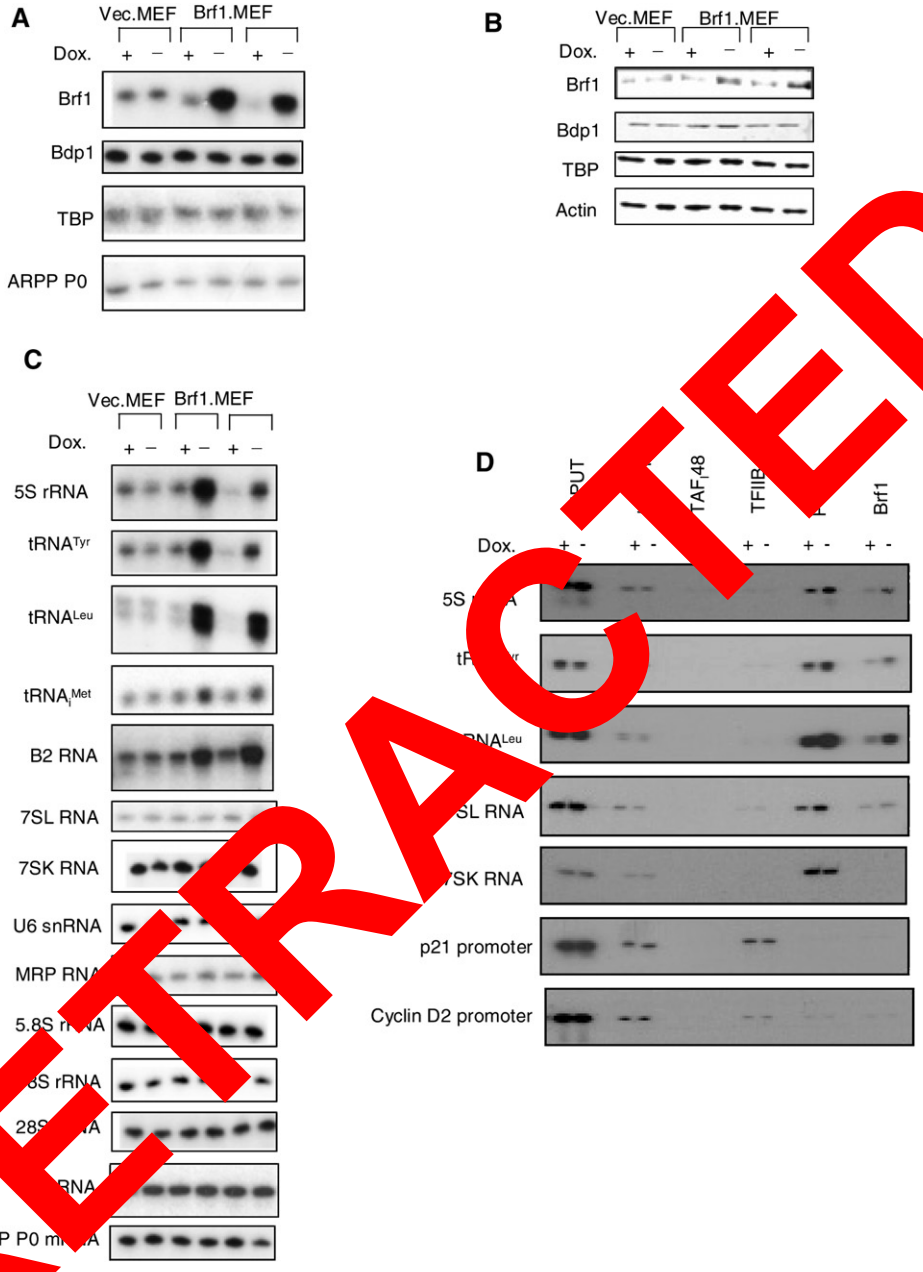


Figure 1. ction of Brf1 Stimulates tRNA and 5S rRNA Expression Selectively

(A) RT-PCR o dicated mRNAs from a vec.MEF clone (lanes 1 and 2) and two Brf1.MEF clones (lanes 3–6) after 48 hr with (odd lanes) or without (even lanes) doxycycline.

(B) Immunoblot of Brf1, Bdp1, TBP, and actin in vec.MEF (lanes 1 and 2) and two Brf1.MEF clones (lanes 3–6) after 48 hr with (odd lanes) or without (even lanes) doxycycline.

(C) RT-PCR of the indicated transcripts from a vec.MEF clone (lanes 1 and 2) and two Brf1.MEF clones (lanes 3–6) after 48 hr with (odd lanes) or without (even lanes) doxycycline.

(D) ChIP assay to test occupancy of TAF_I48 (lanes 5 and 6), TFIIB (lanes 7 and 8), pol III (lanes 9 and 10), and Brf1 (lanes 11 and 12) at the indicated genes in Brf1.MEFs after 48 hr with (odd lanes) or without (even lanes) doxycycline. Input lanes show product intensities obtained using 10% (lanes 1 and 2) or 1% (lanes 3 and 4) of input.

(Anand et al., 2002; Donze et al., 1995; Lazaris-Karatzas et al., 1990; Mayeur and Hershey, 2002). We therefore investigated if Brf1 has a similar effect.

Equal numbers of Brf1.MEF or vec.MEF cells were plated in the presence or absence of doxycycline. Initial rates of proliferation were similar in all cases, but after 3 days the cells with elevated Brf1 began to proliferate faster than the other populations. By 5 days after plating in the absence of doxycycline, the number of Brf1.MEFs had reached approximately twice that seen in the presence of doxycycline or with vec.MEF controls (Figure 3A).

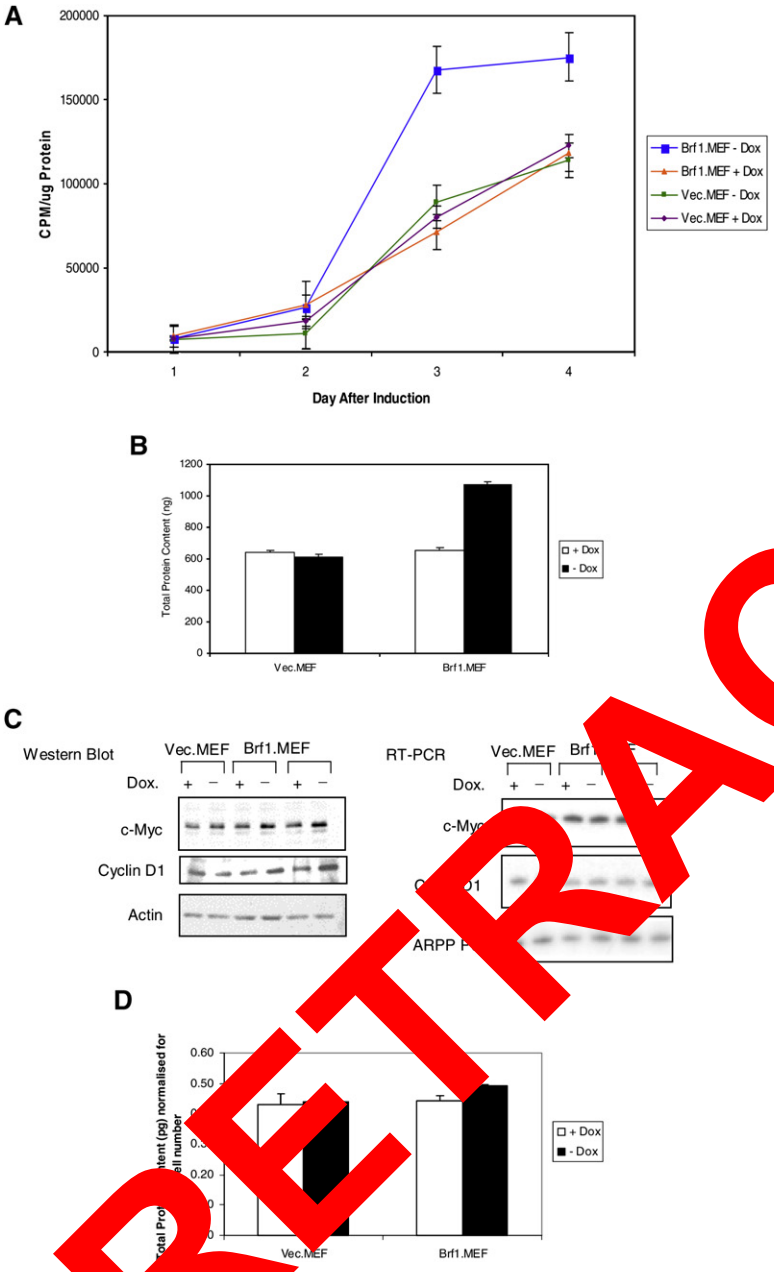


Figure 2. Induction of Brf1 Stimulates Protein Synthesis

(A) Incorporation of ^{35}S-labeled methionine and cysteine into newly synthesized polypeptides in Brf1.MEFs and vec.MEF controls. Values are means ± standard deviation of three separate experiments, each with three replicates per condition.

(B) After 4 days with or without doxycycline, total protein content was measured for vec.MEF and Brf1.MEF cultures. Values are means ± standard deviation of three separate experiments.

(C) Immunoblot of protein lysates (left panel) and RT-PCR of mRNA levels (right panel) for cyclin D1, c-Myc, and actin in vec.MEF (lanes 1 and 2) and two Brf1.MEF clones (lanes 3–6) after 48 hr with (odd lanes) or without (even lanes) doxycycline.

(D) After 4 days with or without doxycycline, total protein content was measured for vec.MEF and Brf1.MEF cultures and adjusted for cell number to give protein/cell. Values are mean ± SD of three separate experiments.

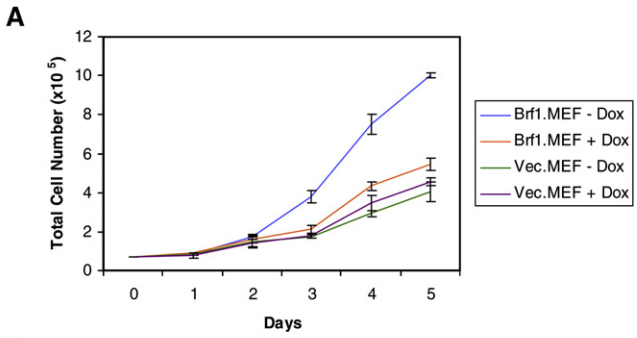
This dramatic effect was not a peculiarity of an individual clone but has been observed with eight independent clones of Brf1.MEFs. It can also be obtained using an adenovirus vector that produces Brf1. Thus, immortalized MEFs infected with Brf1-expressing adenovirus proliferate more rapidly than the same MEFs infected with a control adenovirus that encodes GFP (Figure 3B).

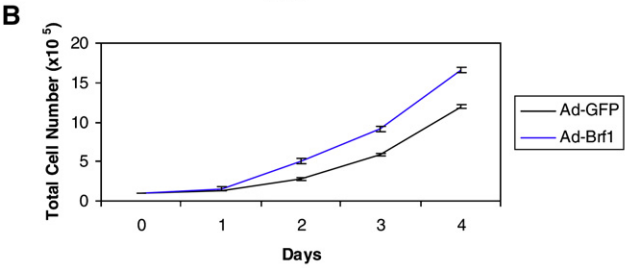
Accelerated rates of cell-cycle progression or diminished rates of cell death might be responsible for the effect of Brf1 induction on population numbers. FACS analysis of relative DNA content was used to examine these features (Figures 3C and S3). Only a small proportion of cells in each population was found to have a sub-G1 content of DNA, which is indicative of apoptosis. Although this fraction was suppressed further when Brf1 was induced, so few cells were apoptotic that this could not account for the overall increase in numbers. Far more substantial changes were seen in the cell-cycle distribution of the viable Brf1.MEFs. Thus, the fraction of cells in G0/G1 phase decreased from 58% to 40% after Brf1 induction, whereas the fraction in S phase increased from 26% to 36%. In contrast, doxycycline withdrawal had little effect on vec.MEF controls. We conclude that Brf1 can stimulate cell-cycle progression.

Brf1 Can Increase Proliferation in Several Cell Types

As with immortalized MEFs, IMR90 human diploid fibroblasts proliferate more rapidly when infected with adenoviral Brf1 expression vector, compared with cells infected with control adenovirus expressing GFP (Figure 4A). The proliferative response to elevated Brf1 is therefore not confined to rodent fibroblasts. The effect is transient with adenovirus due to rapid depletion of exogenous Brf1 from proliferating cells (Figure 4A, lower panels).

To determine if this response can also be obtained in epithelial cells, we used the Tet-Off system to produce stably transfected lines of CHO cells in which expression of exogenous Brf1 can be controlled with doxycycline (Figure 4B). As in MEFs, mild induction of Brf1 in CHO cells selectively raises expression of a subset of pol III transcripts, including tRNA and 5S rRNA (Figure 4C). Furthermore, it again exerts a strong stimulatory effect on cell proliferation (Figure 4D). Four independent clones of Brf1.CHO cells were tested in this way, and in each case doxycycline withdrawal was found to confer a marked proliferative advantage. We conclude that elevated levels of the pol III transcription factor Brf1 can be sufficient to drive proliferation of mammalian fibroblasts or epithelial cells.





C

Cell Line	Vec.MEF (Fraction of cell cycle %)		Brf1.MEF (Fraction of cell cycle %)	
Dox	+	-	+	-
Sub G_1	1.4	1.2	1.4	1.0
G_0/G_1	58.8	57.7	57.5	
S	25.8	26.9	25.5	
G_2/M	14.0	14.2		23.3

Figure 3. Brf1 Can Stimul Cell Prolifer

(A) Cell number counts o days after plating 1.MEFs or vec.MEFs cultured in the presen absen f doxycycline. Mean cell numbers are plotted ± SD for th depen vec.MEF clones and four independent Brf1.MEF clones.

(B) Cell numb unts ov days aft jection of MEFs with adenovirus expressin P. Mean s are plotted ± SD for three independent ex ments.

(C) P ve prop ns of MEFs in different cell-cycle phases 48 hr after plating, t ytometry of relative DNA content.

Activation Pol III Transcription Is Required for Brf1 to S late Proliferation

As part of TFIIIB, Brf1 associates with several regulatory factors that can have a profound influence on cell proliferation, including p53, RB, TBP, c-Myc, and Erk (Felton-Edkins et al., 2003b; White, 2005). This raises the possibility that the effects of Brf1 induction may reflect sequestration of one or more of these key regulators. This is unlikely to be the case for p53 because it contacts the TBP subunit of TFIIIB rather than Brf1 (Crighton et al., 2003). Overexpressing Brf1 might have been predicted to sequester TBP away from SL1 and TFIID complexes, thereby reducing transcription by pols I and II. However, the constant expression of several mRNAs and rRNAs seen in Figure 1B indicates that the Brf1 levels are insufficient to produce such effects. Brf1 is bound directly by c-Myc, Erk, and CK2, but sequestration of any of these mitogenic proteins would be expected to inhibit rather than stimulate proliferation. In contrast, sequestration of RB might be predicted to produce this response. Since RB interacts with Brf1 (Chu et al., 1997; Hirsch et al., 2004; Larminie et al., 1997), we investigated expression of the E2F-regulated genes encoding DH and p107, which are known to be repressed h e RB f in MEFs (Hurford et al., 1997). However, the NAs encod by these genes did not respond to ind tion f1 (Figu 6A). This suggests that levels of Brf1 in clones a uffi t to trigger a general deregulation B ta t gene though more selective changes can e ed.

Brf1 is considere be l-specif ranscription factor, but we cannot e de the po ility t t has additional functions that rem e discovere herefore tested if the ability of Brf1 t e p ration requi s its well-characterized role in pol III transcription. 39 is a specific subunit of pol III that interac Brf1 in order cruit the polymerase to its genetic te ates (Schramm and Hernandez, 2002). RNAi was used to re ce the level RPC39 in Brf1.MEFs. In the presence of doxycycline (Brf1 not uced), partial depletion of RPC39 in this way had imal eff on tRNA expression or proliferation, implying that R in relative excess (Figure 5B). However, when PC39 levels are restricted in this way, there is insufficient active upport increased tRNA expression after withdrawal of oxycycline and the proliferative response to Brf1 induction is blocked (Figure 5C). This is unlikely to be due to any off-target effect, as the same response was obtained with alternative siRNAs against different parts of the RPC39 sequence. These data provide evidence that Brf1 cannot stimulate cell proliferation when pol III transcription is prevented from rising.

Brf1 Induction Can Transform Cells

Induction of Brf1 is accompanied by morphological changes that are often seen in transformed cells, as well as focus formation and increased saturation density (Figures 6A and 6B). Such changes were not observed with empty vector control clones. To test for anchorage dependence, we assayed for colony formation in soft agar (Figures 6C and S4). As expected, vec.MEF and vec.CHO controls produced no macroscopic colonies. In contrast, Brf1.MEF and Brf1.CHO clones were able to form colonies, but only in the absence of doxycycline. Brf1 induction can therefore compromise contact inhibition and allow anchorage-independent colonies to form.

Since these observations suggest that Brf1 can have transforming properties, we tested for tumor formation in mice. Two independent clones of Brf1.MEFs were injected into mice, in parallel with two independent vec.MEF clones and a Ras-transformed line as positive control. Both Brf1.MEF clones caused tumors, albeit less rapidly than the Ras-transformed positive control (Figure 6D). All 15 mice that received Brf1.MEFs developed tumors within 3 months of injection. In contrast, none of the 10 mice injected with vec.MEF control cells had tumors after 6 months, when the experiment was terminated. These data provide evidence that elevated Brf1 levels can cause oncogenic transformation in vivo.

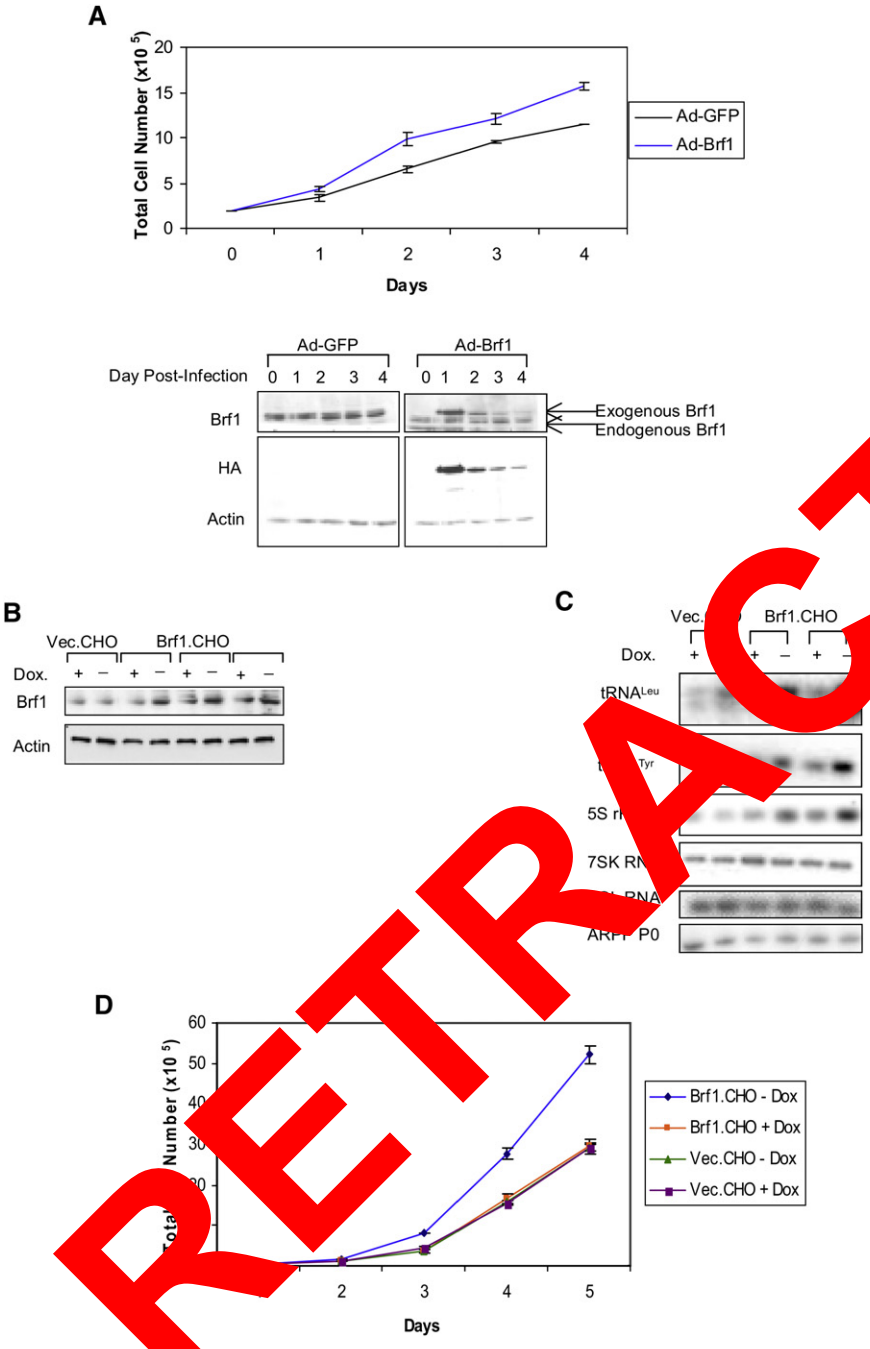


Figure 4. Brf1 Can Stimulate the Proliferation of Several Cell Types

(A) Cell number counts over 4 days after infection of IMR90 fibroblasts with adenovirus expressing HA-tagged Brf1 or GFP. Mean cell numbers are plotted ± SD for two independent experiments. An immunoblot probed with antibodies against Brf1 (upper panel), HA, and actin (lower panel) is shown underneath.

(B) Immunoblot to co[illegible]re levels [illegible]1 and actin in extracts of vec[illegible] (lanes 1 and [illegible]nd three Brf1.CHO clones ([illegible]3–8) after 4[illegible]r in the presence ([illegible]anes) [illegible]ence (ev[illegible]anes) of doxycyc[illegible]

(C) R[illegible]CR anal[illegible]s of lev[illegible]the indicated tra[illegible]pts in [illegible]c.CHO clone[illegible] (lanes 1 and 2) and t[illegible]HO clone[illegible]anes 3–6) after 48 hr [illegible] the p[illegible]ce (odd[illegible]es) or absence (even lanes) of do[illegible]li[illegible]

(D) Cell numbe[illegible]ts over 5 days after plating of [illegible] CHOs or vec.CHOs cultured in the presence o[illegible]ence of doxycycline. Mean cell numbers are p[illegible]d ± standard deviation for three independent v[illegible]c.CHO clones and three independent Brf1.CHO clones.

We also tested the influence of Brf1 on a well-characterized model of tumorigenesis. Loss of p53 is a common step in the development of colon cancer (Kinzler and Vogelstein, 1996). This can be mimicked using the HCT116 colon carcinoma cell line and a derivative with targeted disruption of both p53 alleles (Bunz et al., 1998). The p53 null cells form large foci that are not observed with the p53-positive cells (Figure 6E). Focus formation in this model can be severely impaired by RNAi-mediated depletion of c-Myc, a key player in colon carcinogenesis (Sansom et al., 2007). Similarly, siRNAs directed against the Brf1 message were found to inhibit focus formation (Figure 6E).

Elevated Expression of $tRNA_i^{Met}$ Can Stimulate Proliferation

Among the targets of Brf1 are the genes encoding $tRNA_i^{Met}$, the tRNA required for polypeptide chain initiation. We constructed clones of immortalized MEFs that carry stably transfected copies of a plasmid containing this gene. RT-PCR confirmed that levels of $tRNA_i^{Met}$ are modestly raised in these clones, without exceeding the physiological range (Figure 7A). The overall rate of translation is significantly elevated in these clones (Figure 7B). As in Brf1.MEFs, expression of c-Myc and cyclin D1 proteins increase without a corresponding change in their mRNAs (Figures 7A and 7C). Raising $tRNA_i^{Met}$ can therefore cause quantitative and qualitative changes in protein expression. These effects are selective, as they are not seen in MEFs treated in the same way with plasmid carrying an elongator $tRNA_e^{Met}$ gene. Counts of cell number revealed that $tRNA_i^{Met}$ elicits a consistent increase in the rate of proliferation (Figure 7D). This effect was seen with each of four independent $tRNA_i^{Met}$.MEF lines. Thus, the proliferative response to Brf1 can be recapitulated by raising the expression of one of its pol III-transcribed targets. MEFs transfected with the $tRNA_e^{Met}$ gene showed no consistent change in proliferation relative to empty vector controls.

We were surprised to observe that $tRNA_i^{Met}$.MEFs form foci (Figure 7E). Two independent clones were therefore

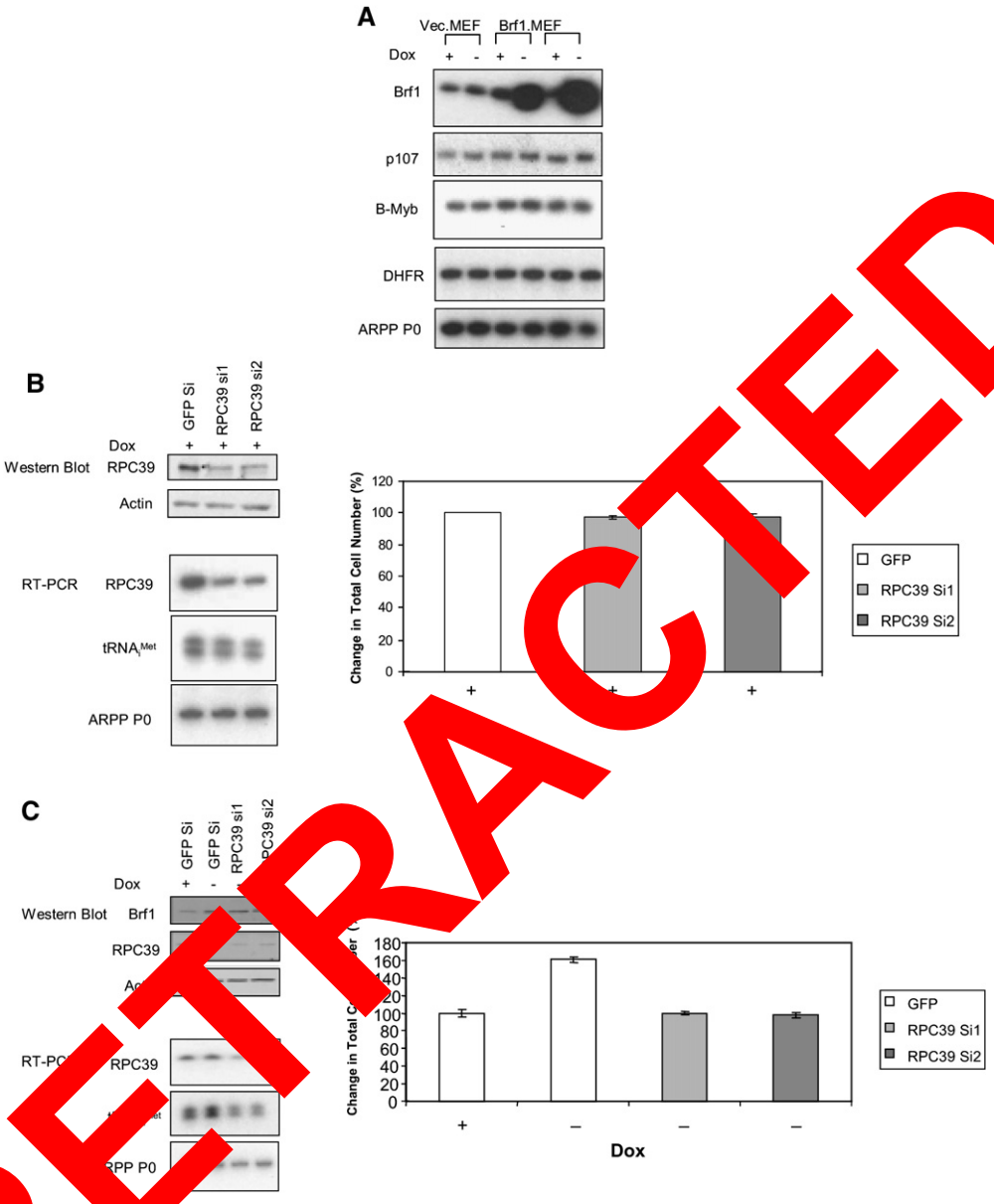


Figure 6. Induction of Proliferation by Brf1 Requires Elevated Pol III Transcription

(A) RT-PCR analysis of the indicated transcripts from a vec.MEF clone (lanes 1 and 2) and two Brf1.MEF clones (lanes 3–6) after 48 hr in the presence (odd lanes) or absence (even lanes) of doxycycline.

(B) Brf1.MEFs were cultured for 5 days in the presence of doxycycline and transfected after 72 hr with the indicated siRNAs. Panels on the left show levels of RPC39 and actin protein, as determined by western blot (upper two panels) and levels of RPC39 mRNA, $tRNA_i^{Met}$, and ARPP P0 mRNA, as determined by RT-PCR (lower three panels). Graph shows mean numbers of viable cells ± SD for two independent experiments.

(C) Brf1.MEFs were cultured for 5 days with (lane 1) or without (lanes 2–4) doxycycline and transfected after 72 hr with the indicated siRNAs. Panels on the left show levels of Brf1, RPC39, and actin protein, as determined by western blot (upper 3 panels) and levels of RPC39 mRNA, $tRNA_i^{Met}$, and ARPP P0 mRNA, as determined by RT-PCR (lower 3 panels). Graph shows mean numbers of viable cells ± standard deviation for two independent experiments.

injected into mice, as were controls carrying Ras or empty vector. Also included was a $tRNA_e^{Met}$.MEF line, which did not show enhanced translation, proliferation, or focus formation. All five mice receiving the Ras-transformed positive control line developed tumors within 4 weeks of injection. At this time, tumors were not visible in any of the other mice. However, tumors began to appear by week 6 in mice carrying $tRNA_i^{Met}$.MEFs, and 9 out of 10 of these mice had tumors by week 12 (Figure 7F). In contrast, no tumors were detected in the 15 mice injected with vec.MEF or $tRNA_e^{Met}$.MEF cells, even after 7 months, when the experiment was terminated. These data provide evidence that elevated expression of $tRNA_i^{Met}$ can confer tumorigenicity on immortalized MEFs.

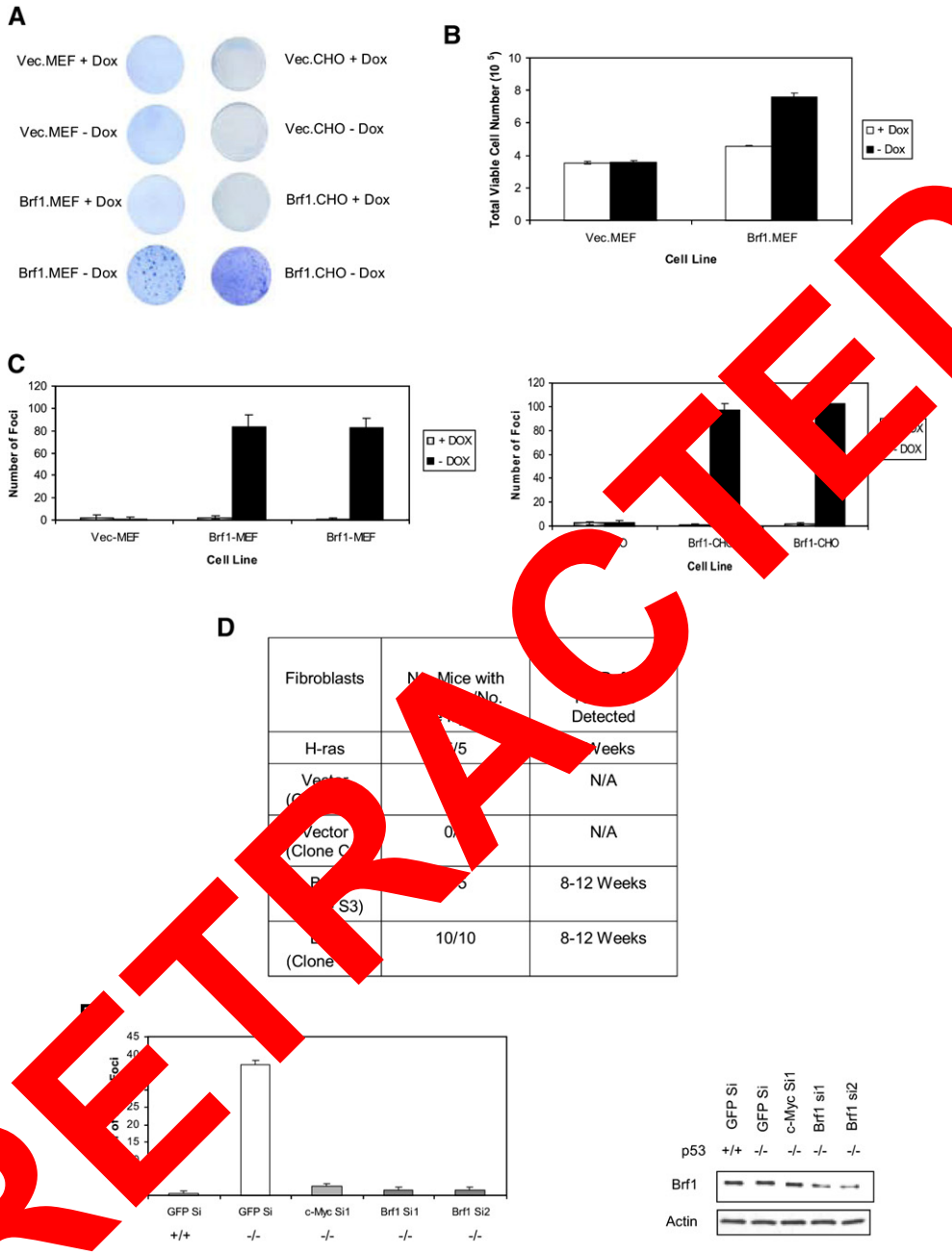


Figure 6. Induction of Brf1 Can Transform Cells

(A) Focus formation of MEF or CHO clones after 3 weeks in the presence or absence of doxycycline, as indicated.

(B) Mean numbers of confluent MEFs ± SD after 3 weeks in the presence or absence of doxycycline. Data are for two independent experiments.

(C) Mean numbers of colonies ± SD formed by a vec.CHO clone, two independent Brf1.CHO clones, a vec.MEF clone, and two independent Brf1.MEF clones cultured for 3–4 weeks in soft agar. Data shown are for three independent experiments.

(D) Mice were injected with Ras-transformed MEFs, two independent vec.MEF clones, and two independent Brf1.MEF clones. The number of mice that developed tumors and the latency of onset are indicated in each case.

(E) Matched $p53^{+/+}$ or $p53^{-/-}$ HCT116 cells were transfected with the indicated siRNAs. Panels on the right show levels of Brf1 and actin protein, as determined by western blot. Graph shows mean numbers of large foci (>3 mm diameter) ± SD for two independent experiments.

DISCUSSION

Overexpression of pol III transcripts has long been associated with cancer (White, 2004). Our data provide evidence that this can, at least in certain contexts, be sufficient to drive proliferation and oncogenic transformation. This has been demonstrated by artificially raising levels of the Brf1 subunit of TFIIIB, which results in enhanced recruitment of pol III to tRNA and 5S rRNA genes

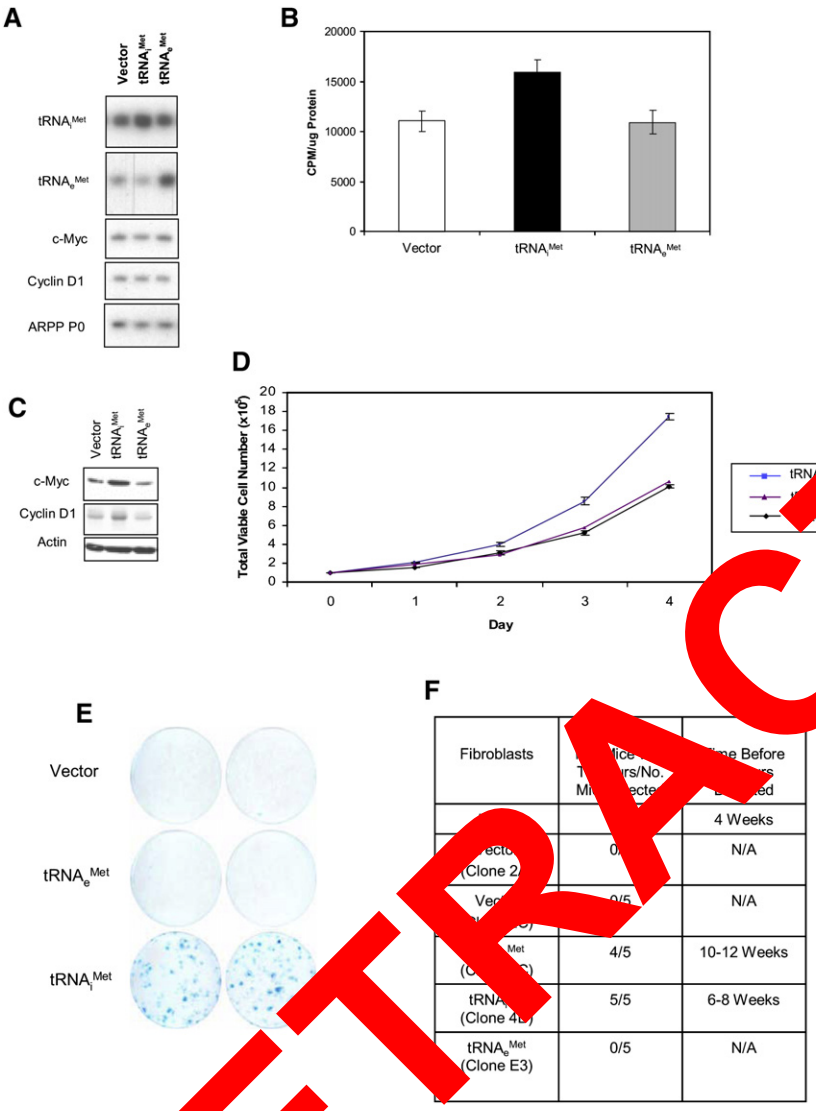


Figure 7. Elevated Expression of $tRNA_i^{Met}$ Can Stimulate Protein Synthesis, Proliferation, and Focus Formation

(A) RT-PCR to compare levels of the indicated transcripts in MEF clones transfected with empty vector (lane 1), $tRNA_i^{Met}$ (lane 2), and $tRNA_e^{Met}$ (lane 3) constructs.

(B) Incorporation of ^{35}S-Met and ^{35}S-Cys into newly synthesized p in MEFs transfected with empt tor, tRN or $tRNA_e^{Met}$. Values are me SD of two s ate experiments, each with independent or clones, four inde ent tR t clones two independe NA_e^{Met} clone

(C) stern b to com evels of c-Myc, D1, a tin proteins i MEF clones transfec pty vecto ne 1), $tRNA_i^{Met}$ (lane 2), and tR et (lane nstructs.

(D) Cell nu s over 4 days after plating of MEF clones t cted with empty vector, $tRNA_i^{Met}$, tRNA$_e^{Met}$ constructs. Values are means ± SD of eparate experiments, each with two independent vector clones, four independent $tRNA_i^{Met}$ clones, and two independent $tRNA_e^{Met}$ clones.

(E) Duplicate focus formation assays with MEF clones transfected with empty vector, $tRNA_i^{Met}$, or $tRNA_e^{Met}$ constructs.

(F) Mice were injected with Ras-transformed MEFs, two independent clones of vec.MEFs, two independent $tRNA_i^{Met}$.MEF clones, and a $tRNA_e^{Met}$.MEF clone. The number of mice that developed tumors and the latency of onset are indicated in each case.

in vivo, thereby in their e ession. Furthermore, the phenotypic ts of B ducti can be mimicked by elevated levels of po roduct . The data suggest that activation of Pol III tra ription, so ten observed in transformed cells, can h fun uences for the development of cancer.

There eady considerable evidence that hyperactivity of the transla machinery can cause proliferation and cell transformation (Bj i and Houghton, 2004; Mamane et al., 2004; Ruggero and Pandolfi, 2003). Most pertinent to our findings is the oncogenicity of eEF1A (formerly known as EF-1α), which controls the recruitment of amino-acylated tRNA to the ribosome (Anand et al., 2002; Tatsuka et al., 1992; Tomlinson et al., 2005). When overexpressed in fibroblasts, eEF1A can stimulate proliferation and allow formation of colonies in soft agar and tumors in nude mice (Anand et al., 2002). Furthermore, it is upregulated in human ovarian and breast carcinomas, often due to gene amplification (Anand et al., 2002; Tomlinson et al., 2005). A tRNA methyltransferase called Misu is overexpressed in breast and colon cancers, and RNAi of Misu inhibits growth of carcinomas (Frye and Watt, 2006). Such observations support the contention that tRNA metabolism can strongly influence oncogenic transformation. Our data provide evidence that elevated tRNA production can have a causal role.

Levels of $tRNA_i^{Met}$ have been shown to have a profound effect on cell proliferation in *S. cerevisiae*, with a 2-fold reduction in expression causing a 3-fold slowing of doubling time (Francis and Rajbhandary, 1990). This can be explained if the availability of $tRNA_i^{Met}$ is limiting for the overall rate of protein synthesis. Our data provide clear evidence that this is indeed the case in mammalian fibroblasts, where modest overexpression of $tRNA_i^{Met}$ was found to stimulate translation significantly. It is well-established that biosynthesis and the attainment of adequate mass are essential prerequisites for cell-cycle progression (Brooks, 1977; Johnston et al., 1977; Sudbery, 2002). Since 80%–90% of a cell's dry mass is protein, the rate of translation is a major determinant of mass accumulation and hence growth and proliferation (Zetterberg and Killander, 1965). In some situations, rapid protein synthesis can result in unbalanced growth and a change in cell size. This is common in yeast and insects, but less so in mammals, where growth and cell division show tighter coupling (Stocker and Hafen, 2000). Indeed, we found only minor effects on cell volume when Brf1 was induced, indicating that the effects on growth and cell-cycle progression are balanced in this case.

In principle, a global increase in protein synthesis may be sufficient to drive cell growth and proliferation. However, such effects might also be due to more selective induction of specific proteins. There is considerable evidence that stimulating translational capacity can cause a preferential increase in the synthesis of growth factors, cell-cycle promoters, and oncoproteins, such as VEGF, cyclin D1, and c-Myc (Mamane et al., 2004). The rationale for this phenomenon is that mRNAs encoding such proteins frequently contain highly structured 5′-untranslated regions; as a consequence, these mRNAs are translated inefficiently and are disproportionately sensitive to rates of protein synthesis, when compared to most mRNAs encoding housekeeping products (Koromilas et al., 1992; Mamane et al., 2004). The proliferative and transforming activities of Brf1 and $tRNA_i^{Met}$ might therefore reflect selective downstream induction of one or more specific proteins. Indeed, we found that Brf1.MEFs and $tRNA_i^{Met}$.MEFs express elevated levels of cyclin D1 and c-Myc proteins without a corresponding change in their mRNAs. These products might contribute substantially to the observed phenotypic effects.

We have used induction of Brf1 as a tool to raise pol III transcription. In so doing, we are mimicking a situation that has been encountered in human cancers. A small study of cervical carcinoma biopsies found that a subset display abnormally high levels of Brf1 mRNA (Daly et al., 2005). Furthermore, ONCOMINE (www.oncomine.org), a web-based microarray database and data-mining platform (Rhodes et al., 2004), identifies two independent studies of bladder and prostate carcinomas in which Brf1 is expressed at elevated levels in tumors relative to normal tissue. In such cancers, Brf1 may be exerting significant proliferative and oncogenic effects, as we have observed in cultured cells. The gene encoding Brf1 is identified as a target for transcriptional induction by c-Myc in colonic epithelium (Sansom et al., 2007). Deregulated Brf1 expression may therefore be a common feature of many tumors in which c-Myc is activated. This raises the possibility of positive feedback since we found transcriptional induction of c-Myc when protein synthesis increases in response to Brf1. Pol III transcription is also stimulated directly by c-Myc (Gomez-Roman et al., 2003).

We have shown that RNAi-mediated knockdown of Brf1 mRNA can inhibit focus formation by colon carcinoma cells. Targeting pol III machinery might therefore have therapeutic potential. It has been suggested that repression of pol III transcription may be important for the tumor suppression function of RB (Chesmyth, 1996; Larminie et al., 1998; Neufeld and Edgar, 1998), but our data provide support for this contention.

EXPERIMENTAL PROCEDURES

Inducible Cell Lines

Expression construct pTRE2-Brf1 contains human Brf1 cDNA cloned into pTRE2 vector (Clontech). The recipient mouse embryonic fibroblast (MEF/3T3) line and Chinese hamster ovary (CHO) Tet-Off cells (Clontech) that express the tetracycline-controlled transactivator were cultured in DMEM or α-MEM, respectively, supplemented with 10% doxycycline-free FCS (Clontech), 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 100 μg/ml G418 sulfate. The MEF/3T3 line was established from primary MEFs by spontaneous immortalization after culture according to the standard 3T3 protocol (3 day transfer, 3 × 10^5 cell inoculum). Cells were transfected using Lipofectamine (Invitrogen) when 70%–80% confluent with 17 μg of pTRE2-Brf1 plus 2 μg of the hygromycin resistance plasmid pTK-Hyg (Clontech). After 48 hr, medium was supplemented with 2 μg/ml doxycycline and 400 μg/ml hygromycin B (Melford Laboratories). After 2–4 weeks, hygromycin-resistant colonies were picked and screened for Brf1 induction. For routine culture, medium was supplemented with 100 μg/ml hygromycin B instead of 400 μg/ml that was used during the selection. To induce Brf1 expression, cells were washed twice in prewarmed PBS and cultured without doxycycline.

Fragments containing $tRNA^{Met}$ genes were [illegible] amplified from mouse genomic DNA with primer pairs 5′-ATCCTTCC[illegible]GCTCATTTTG[illegible]nd 5′-GC GCTTCGAGATGTTTTCATC-3′ for $tRNA_i^{Met}$ and [illegible]CCATTGTGT[illegible]GTTGAT GG-3′ and 5′-GTCCAGGTCCGCTTAG[illegible]SAC-3′ [illegible]RNA_e^{Met}. These fragments were cloned into p-GEM-T E[illegible]vector (Prom[illegible]Sta[illegible]. Transfected MEFs were established as above except with[illegible]t doxyc[illegible]

Adenovirus-Mediated Brf1 Ex[illegible]

GFP-tagged replication[illegible]cient ad[illegible]irus exp[illegible]ing HA-tagged human Brf1 was constructe[illegible]d produced as [illegible]ic[illegible] (Goodfellow et al., 2006). MEF/3T3 cells w[illegible]d at an moi of [illegible] 8 hr, after which the culture medium was re[illegible]ced. In[illegible]ofluorescence analysis confirmed that transduced genes were expresse[illegible]95% of cells within 36 hr.

Immu[illegible]blotting

Cell[illegible]ates were prepared and immunoblotting performed as previously (Go[illegible]llow et al., 2[illegible] White et al., 1995) using antibodies 58C9 against TBP, [illegible]against HA[illegible]10 against c-Myc, 72-13G against cyclin D1, C11 against[illegible](Santa[illegible]z Biotechnologies), 482 and 128 against Brf1 (Cairns and White, [illegible]563 against Bdp1 (Fairley et al., 2003), and C39 against [illegible]39 (Jones et al., 2000). Antibody 482 was raised by immunizing rabbits w[illegible]is peptide IDDLEIDRYILNESE (residues 452–466 of human Brf1).

RT-PCR Analysis

RNA was extracted using TRI reagent (Sigma), according to the manufacturer's specifications. Reverse transcription was for 1 hr at 42°C with 1 μg of RNA, 200 ng of random hexamers (Promega), and 400U of reverse transcriptase (Life Technologies) in 40 μl of 1× First Strand Buffer (Life Technologies) containing 10 mM DTT and each dNTP at 0.5 mM. CDNA (1 μl) was amplified in the presence of 1.85 μCi of [α-^{32}P] dCTP using 20 pmol of primers. Primers and amplification conditions are described in the Supplemental Data.

ChIP Assays

ChIP was performed as previously using published primers and amplification procedures (Fairley et al., 2005; Gomez-Roman et al., 2003). Antibodies were M19 against TAF_I48 and C-18 against TFIIB (Santa Cruz Biotechnologies), 128 against Brf1 (Cairns and White, 1998), and 1900 against RPC155 (Fairley et al., 2003). Serial dilutions of input chromatin were used to establish that PCRs were within a linear range.

Translation, Apoptosis, Proliferation, and Cell-Cycle Analysis

To measure protein synthesis, 2 × 10^3 cells were inoculated into each well of a 24-well plate. 5 μCi of ^{35}S-Met and ^{35}S-Cys was added per ml of medium 1 hr before harvesting; incorporation of radiolabel into acid-insoluble material was determined, as described (Goodfellow et al., 2006).

To measure proliferation, 7 × 10^4 cells were inoculated into 10 cm dishes in the presence or absence of 2 μg/ml doxycycline. Medium and doxycycline were renewed every 48 hr. Numbers of viable cells were counted each day after trypan blue (Sigma) staining.

For cell-cycle analysis, MEFs were plated at a density of 10^5 cells per 10 cm dish and cultured for 48 hr before washing twice in PBS and harvesting with 5 ml cell dissociation buffer (Sigma). Cells were pelleted by centrifuging at 1000 rpm for 3 min, washed twice in PBS, and then fixed by adding 1 ml of ice-cold 1:1 (v/v) PBS/ethanol in a drop-wise fashion, with gentle agitation. On the day of analysis, fixed cells were washed twice with PBS, resuspended in RNase A (Sigma, 50 μg/ml) and propidium iodide (PI, Sigma, 10 μg/ml), and incubated in the dark at room temperature for 1 hr before flow cytometric sorting (Beckmann).

RNAi

MEFs were transfected at 40% confluence to a final concentration of 50 nM siRNA oligonucleotides, delivered using Lipofectamine 2000 (Invitrogen). Medium was replaced after 5 hr and cells were harvested after a further 48 hr. SiRNAs were all from Ambion: RPC39 si1 5′-GGAAAAACUGGUCUAUCAA-3′; RPC39 si2 5′- GGAUUCUCAGAAUGCUGGU-3′; GFP (cycle 3); c-Myc 5′-GGAGGAACGAGCUAAAACG-3′; Brf1 si1 5′-GGAAGAUCUGUUGUUACUU-3′; Brf1 si2 5′-CCCGUGCCUGUAUAUUCCA-3′.

Transformation Assays

For focus formation, cells were seeded at a density of 3×10^5 per 10 cm plate and then grown to confluency in the presence or absence of doxycycline. Cultures were maintained for a further 3 weeks after reaching confluency, with the media changed every 4 days. After 3 weeks, the cells were washed twice with PBS and once with ice-cold methanol and then fixed for 10 min in methanol before being stained with 0.1% (w/v) methylene blue (Sigma) and photographed.

To measure saturation density, cells were seeded as above, cultured for 24 hr before withdrawal of doxycycline, as appropriate, and then grown to confluency. Cell numbers were determined after trypsinization by counting in a haemocytometer.

To assay anchorage-independent growth, 5×10^3 cells in 0.35% (w/v) agar containing 20% (v/v) FCS were overlaid onto 0.5% (w/v) agar in a 35 mm plate. Plates were overlaid with 0.35% (w/v) agar with or without 2 μg/ml doxycycline every 2 days. Colonies were stained with 0.005% (w/v) crystal violet and then photographed.

To measure tumorigenic potential in vivo, MEFs were injected into athymic mice. A total of 2×10^6 cells from each MEF clone were suspended in PBS Buffered Saline (Sigma) and injected subcutaneously into each flank of an animal. After injection, mice were monitored three times/week for tumor formation. Once detected, tumor dimensions were measured three times/week with callipers. Animals with tumor volumes in excess of 1200 mm^3 were sacrificed.

HCT116 cells were plated at a density of 7×10^5 cells per 10 cm dish in McCoy's 5A (GIBCO) supplemented with 10% FBS (Sigma), 2 mM L-glutamine, 100 U/ml streptomycin, and 100 U/ml penicillin. After 24 and 5 days they were transfected with siRNAs to a final concentration of 50 nM and were prepared for immunoblotting after a further 2 days. Staining for foci was carried out as above, 14 days after plating.

SUPPLEMENTAL DATA

Supplemental Data include Experimental Procedures, References, and four figures and can be found with this article online at http://www.cell.com/cgi/content/full/133/1/79/DC1/.

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